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(54) Title: *MYCELIOPHTHORA* AND *SCYTALIDIUM* LACCASE VARIANTS HAVING IMPROVED STABILITY

(57) Abstract

The present invention relates to laccase mutants with improved stability properties, in particular to *Myceliophthora* and *Scytalidium* laccase variants comprising mutations in one or more tyrosine, tryptophan or methionine residues.

MYCELIOPHTHORA AND *SCYTALIDIUM* LACCASE VARIANTS HAVING IMPROVED STABILITY

FIELD OF THE INVENTION

The present invention relates to laccase mutants with improved stability properties.

BACKGROUND OF THE INVENTION

Laccase is a polyphenol oxidase (EC 1.10.3.2) which catalyses the oxidation of a variety of inorganic and aromatic compounds, particularly phenols, with the concomitant reduction of molecular oxygen to water.

Laccase belongs to a family of blue copper-containing oxidases which includes ascorbate oxidase and the mammalian plasma protein ceruloplasmin. All these enzymes are multi-copper-containing proteins.

Because laccases are able to catalyze the oxidation of a variety of inorganic and aromatic compounds, laccases have been suggested in many potential industrial applications such as lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, hair colouring, and waste water treatment. A major problem with the use of laccases are their poor storage stability at temperatures above room temperature, especially at 40°C.

In Example 1 of the present application we have tested the stability of *Myceliophthora thermophila* laccase at 40°C, and it can be seen that after 2 weeks of storage the laccase activity is down to less than 50% of the initial value. For many purposes such a decrease is unacceptable, so it is the purpose of the present invention to create laccase variants with improved stability.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to laccase variants, in particular to
a variant of a parent laccase, which variant has laccase activity, improved stability as compared to said parent laccase,

and comprises a mutation in one or more tyrosine, tryptophan or methionine residues, wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccas has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

In still further aspects the invention relates to DNA encoding such variants and methods of preparing the variants.

Finally, the invention relates to the use of the variants for various industrial purposes.

DETAILED DISCLOSURE OF THE INVENTION

Homologous Laccases

A number of laccases produced by different fungi are homologous on the amino acid level. For instance, when using the homology percent obtained from UWGCG program using the GAP program with the default parameters (penalties: gap weight=3.0, length weight=0.1; WISCONSIN PACKAGE Version 8.1-UNIX, August 1995, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) the following homology was found:

Myceliophthora thermophila laccase comprising the amino acid sequence shown in SEQ ID No. 1: 100%;
Scytalidium thermophilum laccase comprising the amino acid sequence shown in SEQ ID No. 2: 81.2%.

Because of the homology found between the above mentioned laccases, they are considered to belong to the same class of laccases, namely the class of "*Myceliophthora*-like laccases".

Accordingly, in the present context, the term "*Myceliophthora*-like laccase" is intended to indicate a laccase which, on the amino acid level, displays a homology of at least 80% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 85% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 90% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase

which, on the amino acid level, displays a homology of at least 95% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 98% to the *Myceliophthora* laccase SEQ ID NO 1.

In the present context, "derived from" is intended not only to indicate a laccase produced or producible by a strain of the organism in question, but also a laccase encoded by a DNA sequence isolated from such strain and produced in a host organism containing said DNA sequence. Finally, the term is intended to indicate a laccase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the laccase in question.

Variants with altered stability

It is contemplated that it is possible to improve the stability of a parent *Myceliophthora* laccase or a parent *Myceliophthora*-like laccase by making variants:

Such a variant has laccase activity, improved stability as compared to said parent laccase, and comprises a mutation in one or more tyrosine, tryptophan or methionine residues. The parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

Preferred positions for mutations in *Myceliophthora thermophila* laccase (SEQ ID No 1) and in *Scytalidium thermophilum* laccase (SEQ ID No 2) are the following:

Myceliophthora thermophila:

M433,
W373,
W136,
Y145,
M480,
Y137,
Y176,
M254, and/or

W507.

Scytalidium thermophilum:

M483,
W422,
W181,
Y190,
M530,
Y182,
Y221,
M300, and/or
M313.

In particular the following mutations in *Myceliophthora thermophila* laccase (SEQ ID No 1) and in *Scytalidium thermophilum* laccase (SEQ ID No 2) are preferred:

A variant of a parent *Myceliophthora thermophila* laccase, which comprises a substitution in a position corresponding to at least one of the following positions in SEQ ID No. 1:

M433 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
W373 A, V, L, I, P, F, M, G, S, T, C, Y, N, Q, D, E, K, R, H;
W136 A, V, L, I, P, F, M, G, S, T, C, Y, N, Q, D, E, K, R, H;
Y145 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
M480 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
Y137 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
Y176 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
M254 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
W507 A, V, L, I, P, F, M, G, S, T, C, Y, N, Q, D, E, K, R, H;

in particular at least one of the following positions in SEQ ID No. 1:

M433 F, V, I, L, Q;
W373 F, H;
W136 F, H;
Y145 F;
M480 F, V, I, L, Q;
Y137 F;

Y176 F;
M254 F, V, I, L, Q; and/or
W507 F, H.

A variant of a parent *Scytalidium thermophilum* laccase, which comprises a substitution in a position corresponding to at least one of the following positions in SEQ ID No. 2:
M483 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
W422 A, V, L, I, P, F, M, G, S, T, C, Y, N, Q, D, E, K, R, H;
W181 A, V, L, I, P, F, M, G, S, T, C, Y, N, Q, D, E, K, R, H;
Y190 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
M530 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
Y182 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
Y221 A, V, L, I, P, F, W, G, S, T, C, M, N, Q, D, E, K, R, H;
M300 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;
M313 A, V, L, I, P, F, W, G, S, T, C, Y, N, Q, D, E, K, R, H;

in particular at least one of following positions in SEQ ID No. 2:
M483 F, V, I, L, Q;
W422 F, H;
W181 F, H;
Y190 F;
M530 F, V, I, L, Q;
Y182 F;
Y221 F;
M300 F, V, I, L, Q; and/or
M313 F, V, I, L, Q.

<u>Methods of preparing laccase variants</u>

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of laccase-encoding DNA sequences, methods for generating mutations at specific sites within the laccase-encoding sequence will be discussed.

Cloning a DNA sequence encoding a laccase

The DNA sequence encoding a parent laccase may be isolated from any cell or microorganism producing the laccase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the laccase to be studied. Then, if the amino acid sequence of the laccase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify laccase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known laccase gene could be used as a probe to identify laccase-encoding clones, using hybridization and washing conditions of lower stringency.

A method for identifying laccase-encoding clones involves inserting cDNA into an expression vector, such as a plasmid, transforming laccase-negative fungi with the resulting cDNA library, and then plating the transformed fungi onto agar containing a substrate for laccase, thereby allowing clones expressing the laccase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method. In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers.

Site-directed mutagenesis

Once a laccase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the laccase-encoding sequence, is created in a vector carrying the laccase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with T7 DNA polymerase and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into laccase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

The random mutagenesis of a DNA sequence encoding a parent laccase may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the laccase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent laccase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the laccase enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent laccase enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step or the screening step being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are fungal hosts such as *Aspergillus niger* or *Aspergillus oryzae*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

<u>Localized random mutagenesis</u>

The random mutagenesis may advantageously be localized to a part of the parent laccase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of

the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above or any other suitable technique known in the art.

Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently be performed by use of aa filter assay based on the following principle:

A microorganism capable of expressing the mutated laccase enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The top filter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the conditions to be used for screening or may be treated during the detection of enzymatic activity.

The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing

agent, e.g., agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

Testing of variants of the invention

The storage stability of *Myceliophthora* variants or *Myceliophthora*-like variants should be investigated at 40°C for 2 weeks at pH 5, 8 and 9.3, respectively. The stability of the parent laccase and the variants may be tested both in a liquid buffer formulation and in a lyophilized form.

According to the invention the residual activity of the variants following two weeks of incubation are then compared to the residual activity of the parent laccase, and variants with an improved stability at either pH 5, 8 or 9.3 are selected.

Laccase activity

In the context of this invention, the laccase activity was measured using 10-(2-hydroxyethyl)-phenoxazine (HEPO) as substrate for the various laccases. HEPO was synthesized using the same procedure as described for 10-(2-hydroxyethyl)-phenothiazine, (G. Cauquil in Bulletin de la Society Chemique de France, 1960, p. 1049). In the presence of oxygen laccases (E.C. 1.10.3.2) oxidize HEPO to a HEPO radical that can be monitored photometrically at 528 nm.

The *Myceliophthora thermophila* laccase was measured using 0.4 mM HEPO in 25 mM Tris-HCl, pH 7.5, 0.05% TWEEN-20 at 30 °C. The absorbance at 528 nm was followed for 200 s and the rate calculated from the linear part of the progress curve.

Expression of laccase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a laccase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a laccase variant of the invention, especially in a fungal host, are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene, the product of which complements a defect in the host cell,

such as one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a laccase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a laccase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a fungal cell.

The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a

method of producing a laccase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The laccase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

<u>Industrial Applications</u>

The laccase variants of this invention possesses valuable properties allowing for various industrial applications, in particular lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, hair dyeing, bleaching of textiles (in particular bleaching of denim as described in WO 96/12845 and WO 96/12846) and waste water treatment. Any detergent composition normally used for enzymes may be used, e.g., the detergent compositions disclosed in WO 95/01426.

The invention is further illustrated in the following examples, which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

<u>Storage stability of the *Myceliophthora thermophila* laccase</u>

The storage stability of the *Myceliophthora thermophila*

laccase was tested for 2 weeks at 40°C at pH 5, 8 and 9.3, respectively. The laccase (1 mg/ml) was dialyzed against 0.1 M sodium acetate, pH 5, or 0.1 M Tris-maleate, pH 8, or 0.1 M Tris-maleate, pH 9.3. Following dialysis the different preparations were poured into two sets of glass vials with screw caps: one for the liquid formulation and the other one for the lyophilized form. After two weeks of incubation the enzyme activity was measured as described above and the residual activity of the enzyme was calculated in percentage using a preparation of *Myceliophthora thermophila* kept at 4°C as a reference.

Table 1 Storage stability of *Myceliophthora thermophila*

pH	Liquid formulation Residual activity (%)	Lyophilized form Residual activity (%)
5.0	<5	<5
8.0	<5	<5
9.3	35	30

EXAMPLE 2

<u>Storage stability of *Myceliophthora thermophila* variants</u>

Laccase activity:

In this Example the *Myceliophthora thermophila* laccase variants were measured using 0.4 mM HEPO in 0.1 M Tris-maleate, pH 7.5, 0.05% TWEEN-20 at 30°C. The absorbance at 528 nm was followed for 200 s and the rate calculated from the linear part of the progress curve.

The storage stability of the *Myceliophthora thermophila* variants were tested for 4 weeks at 40°C at pH 5, 7, and 9.3, respectively. The laccase (1 mg/ml) was dialyzed against 0.1 M Tris-maleate, pH 5 or 0.1 M Tris-maleate, pH 7 or 0.1 M Tris-maleate, pH 9.3. Following dialysis the different preparations were poured into two set of glass vials with screw caps: one

for the liquid formulation and the other set of glasses for lyophilization. Following two and four weeks of incubation the enzyme activity was measured as described above and the residual activity of the variants were calculated in percentage using a preparation kept at 4°C as reference.

Table 2. Storage stability of *Myceliophthora thermophila* variants, lyophilized formulation

	Residual activity, pH 5		**Residual activity, pH 7**		**Residual activity, pH 9.2**	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
wt	18	18	55	36	59	38
W136F	<5	<5	76	64	88	77
Y137F	12	<5	58	41	64	49
Y145F	<5	<5	53	20	45	51
W373F	14	14	33	19	51	36
M433I	7	<5	57	43	74	35
M480L	33	18	65	32	72	52
W507F	18	<5	72	51	68	71

In lyophilized form none of the tested variants have improved stability at pH 5. At pH 7 and pH 9.2 both W136F and W507F have increased stability. At pH 9.2 M480L is also better than wt.

Table 3. Storage stability of *Myceliophthora thermophila* variants, liquid formulation

	Residual activity, pH 5, 2 weeks	**Residual activity, pH 7, 2 weeks**	**Residual activity, pH 9.2, 2 weeks**
wt	<5	5	20
W136F	5	28	55

Y137F	<5	<5	<5
Y145F	<5	<5	<5
W373F	<5	40	<5
M433I	8	40	65
M480L	<5	<5	15
W507F	<5	<5	22

Also in the liquid formulation none of the tested variants have improved stability at pH 5. At pH 7 and pH 9.2 both W136F and M433I has increased stability. At pH7 W373F has better stability than wt but the variant looses the stability completely at pH 9.2.

Of the tested variants only W136F has increased stability in both formulations.

CLAIMS

1. A variant of a parent laccase, which variant has laccase activity, improved stability as compared to said parent laccase, and comprises a mutation in one or more tyrosine, tryptophan or methionine residues, wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

2. A variant according to claim 1, which comprises a mutation in a position corresponding to at least one of the following positions in SEQ ID No. 1:
M433,
W373,
W136,
Y145,
M480,
Y137,
Y176,
M254, and/or
W507.

3. A variant according to claim 1, wherein the parent laccase is derived from *Myceliophthora*.

4. A variant according to claim 1, wherein the parent laccase is derived from *Scytalidium*.

5. A variant according to claim 4, wherein the parent laccase is a *Scytalidium thermophilum* laccase with the sequence ID No. 2.

6. A variant according to claim 5, which comprises a mutation in a position corresponding to at least one of the following positions in SEQ ID No. 2:
M483,

W422,
W181,
Y190,
M530,
Y182,
Y221,
M300, and/or
M313.

7. A DNA construct comprising a DNA sequence encoding a laccase variant according to any of claims 1-6.

8. A recombinant expression vector which carries a DNA construct according to claim 7.

9. A cell which is transformed with a DNA construct according to claim 7 or a vector according to claim 8.

10. A cell according to claim 9, which is a microorganism.

11. A cell according to claim 10, which is a bacterium or a fungus.

12. A cell according to claim 11, which is an *Aspergillus niger* or an *Aspergillus oryzae* cell.

13. Use of a laccase variant according to any of claims 1-6 for oxidizing a substrate.

14. Use of a laccase variant according to claim 13 for dye transfer inhibition.

15. Use of a laccase variant according to claim 13 for bleaching textiles, in particular for bleaching denim.

16. A detergent additive comprising a laccase variant according to any of claims 1-6 in the form of a non-dusting granulate, a stabilised liquid or a protected enzyme.

17. A detergent additive according to claim 16, which additionally comprises one or more other enzyme such as a protease, a lipase, an amylase, and/or a cellulase.

18. A detergent composition comprising a laccase variant according to any of claims 1-6 and a surfactant.

19. A detergent composition according to claim 18 which additionally comprises one or more other enzymes such as a protease, a lipase, an amylase and/or a cellulase.